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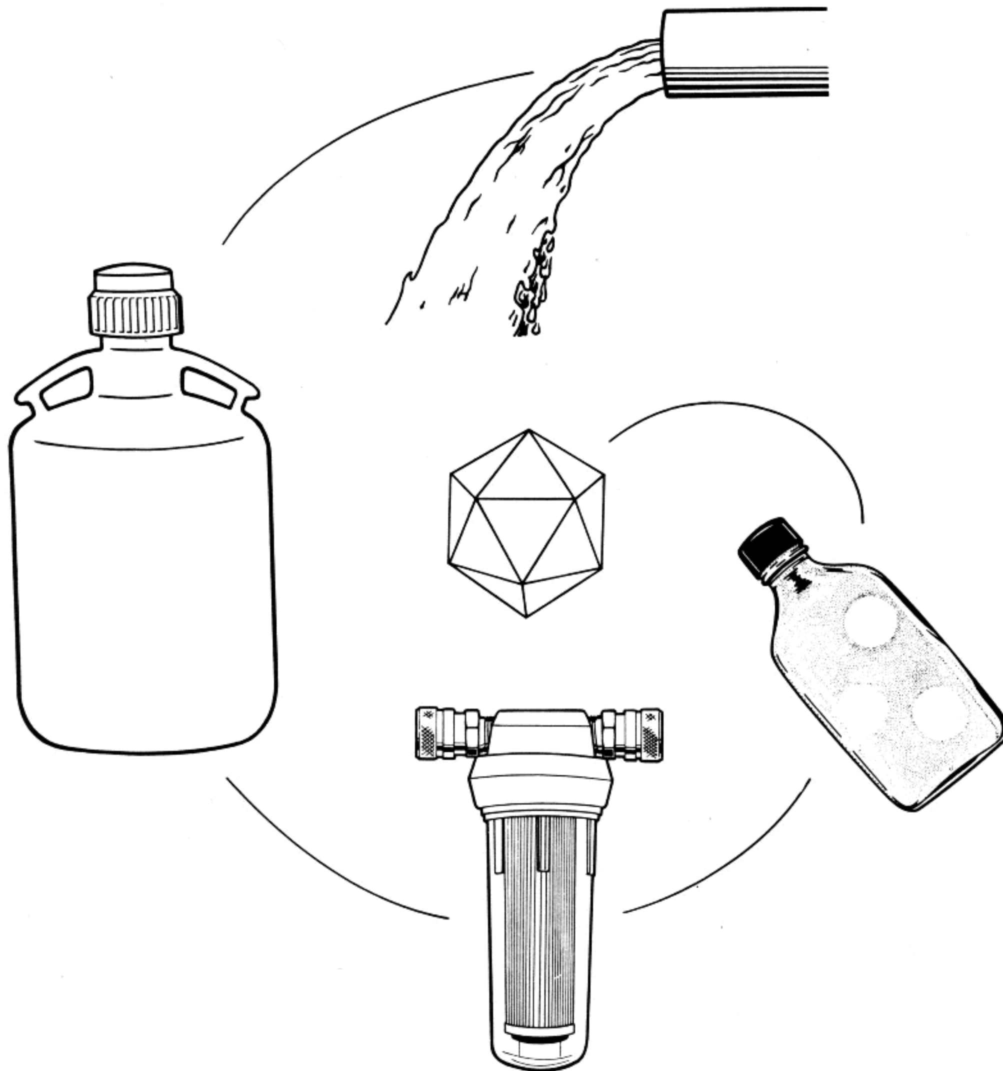
USEPA Manual of Methods for Virology

Chapter 15
April 2001



Chapter 15
TOTAL CULTURABLE VIRUS QUANTAL ASSAY[1]

1. Introduction

1.1 Scope

This chapter describes a quantal method for assaying culturable human enteric viruses from water matrices. The assay differs from the plaque assay described in Chapter 10 (December 1987 Revision) in that it is based upon the direct microscopic viewing of cells for virus-induced cytopathic effects. The quantal method can be used to assay viruses concentrated from sewages, effluents and waters using the methods described in Chapters 5, 6 and 14 (February 1999), Dahling and Wright (1986b), Fout *et al*. (1996) or from sludges and other solids (Chapter 7, September 1989 Revision; Fout, 1999).

1.2 Significance

Quantal assays are less quantitative but more sensitive than plaque assays. In plaque assays each plaque-forming unit (PFU) is counted as developing from a single infectious virus. In quantal assays a positive response may be caused by one or more infectious virus particles and thus quantitation is accomplished through inoculation of replicate samples and the use of most probable number statistics (Chang *et al.*, 1958).

The disadvantage caused by the loss in accuracy in the measurement of infectious virus particles is often offset in quantal assays by an increased sensitivity in the assay's capacity to detect infectious viruses (Morris and Waite, 1980). This increase is presumably due to enhanced replication of some virus strains under quantal assay conditions. Detection of slow growing virus strains is probably also enhanced by quantal assays, because the cell monolayers stay viable for longer periods of time.

1.3 Safety

Human pathogenic enteric viruses can be present in surface or groundwaters impacted by untreated or inadequately treated domestic wastes. The presence of these viruses can cause hepatitis, gastroenteritis and numerous other diseases. Laboratories performing virus analyses are responsible for establishing an adequate safety plan and must rigorously follow the guidelines on decontamination and waste disposal given in Chapter 2 (May 1991 Revision).

2. Apparatus, Materials, Media and Reagents

Analytical Reagent or ACS grade chemicals (unless specified otherwise) and deionized or distilled reagent grade water (dH_2O) should be used to prepare all media and reagents. The dH_2O must have a resistance of greater than 0.5 megohms-cm at 25°C, but water with a resistance of 18 megohms-cm is preferred.

Appendix 1 gives a list of potential vendors for the apparatus, materials, media and reagents used in this method. Equivalent items from other vendors may be substituted for those given in the text.

2.1 Apparatus and Materials

2.1.1 Incubator capable of maintaining the temperature of cell cultures at 36.5 ± 1°C.

2.1.2 Sterilizing filter — 0.22 µm (Costar Product No. 140666).

Always pass about 10 - 20 mL of 1.5% beef extract, pH 7.0-7.5, through the filter just before use to minimize virus adsorption to the filter (see Section 2.2.2).

2.1.3 Sterilizing filter unit — 0.2 µm (PGC Scientifics Product No. 33-8803-42)

2.2 Media and Reagents:

2.2.1 Sodium hydroxide (NaOH) — prepare 1 M and 5 M solutions by dissolving 4 g or 20 g of NaOH in a final volume of 100 mL of dH_2O, respectively.

NaOH solutions are self-sterilizing and may be stored for several months at room temperature.

2.2.2 Beef extract, desiccated powder (Difco Product No. 0115-17-3) — prepare buffered 1.5% beef extract by dissolving 7.5 g of beef extract powder and 1.875 g of glycine (final glycine concentration = 0.05 M) in 450 mL of dH_2O. Adjust the pH to 7.0-7.5 with 1 or 5 M NaOH and bring the final volume to 500 mL with dH_2O. Autoclave at 121°C for 15 min and use at room temperature.

Beef extract solutions may be stored for one week at 4°C or for longer periods at -20°C.

2.2.3 Iodine disinfectant, 0.5% — dissolve 5g of iodine in 1000 mL of 70% ethanol.

2.2.4 Earle's Balanced Salt Solution with 0.5% lactalbumen hydrolysate (ELAH) — dissolve 5 g of lactalbumen hydrolysate (Life Technologies Product No. 11800) and the salts from a 1 L packet of Earle's Balanced Salts (Life

[1]Prepared by G. Shay Fout, D.R. Dahling and R.S. Safferman

Technologies Product No. 81100) in a total volume of 1 L of dH_2O. Sterilize through a sterilizing filter unit and store at room temperature.

2.2.5 Prepare growth medium by supplementing MEM/L-15 medium with 10% serum and antibiotics (see chapter 9 (January 1987 Revision) for recipes to prepare medium and antibiotics; e.g., 100 mL of serum, 1 mL of penicillin-streptomycin stock, 0.5 mL of tetracycline stock and 0.2 mL of fungizone stock per 900 mL of MEM/L-15).

2.2.6 Sterile fetal calf, gamma globulin-free newborn calf or iron-supplemented calf serum, certified free of viruses, bacteriophage and mycoplasma.

Test each lot of serum for cell growth and toxicity before purchasing in quantity. Serum should be held at -20°C for long-term storage. Upon thawing, each bottle must be heat-inactivated in a waterbath set at 56 ± 1°C for 30 min and stored at 4°C for short term use.

2.2.7 Prepare maintenance medium by supplementing MEM/L-15 with antibiotics and 2% serum (20 mL of serum, antibiotics as above for growth medium and 80 mL of dH_2O).

2.2.8 Prepare BGM cell culture test vessels using standard procedures given in Chapter 9 (January 1987 Revision).

The BGM cell culture line is currently the most widely used line for detecting total culturable viruses from water matrices. It is a continuous cell line derived from African Green monkey kidney cells that was first described for clinical use by Barron et al. (1970). The use of BGM cells for recovering viruses from environmental samples and conditions for maximal sensitivity for virus detection have been described by Dahling et al. (1974), Dahling et al. (1984), Dahling and Wright (1986a) and given in Chapter 9 (January 1987 Revision). Although the line is highly susceptible to many enteric viruses, -other cell lines (e.g., RD, MA104, FRhK-4) may be used to increase detection sensitivity.

*Stock cultures of BGM cells may be maintained on roller bottles (**Figure 15-1**). BGM cells to be used for virus detection should be between passage number 117 (the earliest passage routinely available) and 250.*

Figure 15-1 BGM Roller Bottle Culture

3. Sample Inoculation and CPE Development

Wipe down surface areas with disinfectant before and after working with cell cultures.

A microbiological biosafety cabinet should be used to process cell cultures. If a hood is not available, cell cultures should be prepared in controlled facilities used for no other purposes. Viruses or other microorganisms must not be transported, handled, or stored in rooms used for cell culture transfer. It is also recommended that a microbiological biosafety cabinet be used to inoculate cell cultures with water sample concentrates.

Cell cultures used for virus assay are generally found to be at their most sensitive level between the third and sixth days after their most recent passage. Those older than seven days should not be used.

3.1 Identify cell culture test vessels by coding them with an indelible marker. Return the cell culture test vessels to a 36.5 ± 1°C incubator and hold at that temperature until the cell monolayer is to be inoculated.

3.2 Decant and discard the medium from cell culture test vessels. Wash the test vessels with ELAH or growth medium without serum using a wash volume of at least 0.06 mL/cm^2 of surface area. Rock the wash medium over the surface of each monolayer several times and then decant and discard the wash medium.

Do not disturb the cell monolayer.

The wash medium can be added to flasks several hours before inoculation and discarded just prior to inoculation.

3.3 Calculate the **Inoculum Volume**.

3.3.1 Determine the volume of the original water or solids sample to be assayed. It is recommended that this volume be ≥1 L for sewage, ≥100 L for surface or recreational waters, ≥1000 L for finished drinking water or untreated groundwaters or the volume which contains ≥5 g of total sludge or other solids. This volume is designated the **Volume of Original Water Sample Assayed** (D). Record the value of D on a Virus Data Sheet (Appendix 2 gives an example of a Virus Data Sheet that contains information that should be recorded on each sample tested).

*In order to have sufficient sample for assay and account for losses on the sides of containers, problems with cytotoxicity, etc., the **Volume of Original Water Sample Assayed** should represent about 33 to 66% of the total sample collected. Thus, the recommended sample volumes for sewage are ≥2 L. Recommended minimum sample volumes for surface or recreational waters are ≥200 L and ≥1500 L for finished drinking water or for untreated groundwater. Recommended maximum sample volumes for source and recreational waters and finished waters/untreated groundwaters are 300 L and 2000 L, respectively.*

3.3.2 Calculate an **Assay Sample Volume**.

(a) Calculate the **Assay Sample Volume** (S) for water and solids samples using the formula:

$$S = \frac{D}{TSV} \times FCSV$$

where TSV is the **Total Sample Volume** [i.e., the total volume in liters of water or sewage sample passed through a 1MDS filter (Chapter 14, February 1999) or through a negatively charged filter (Chapters 5-6) or of virus concentrates from sludges, soils, sediments and other solids (Chapter 7, September 1989 revision; Fout, 1999)]. FCSV is the **Final Concentrated Sample Volume** [the volume in milliliters of the final processed sample that will be assayed for viruses. This is usually the concentrate from an organic flocculation concentration procedure (e.g., Chapter 14, Step 4.2.7). The **Assay Sample Volume** is the volume of the final concentrated Sample that represents the volume of original water sample assayed. Record the **Assay Sample Volume** onto the Virus Data Sheet.

(b) Calculate the **Assay Sample Volume** for QC and PE samples using the formula:

$$S = 0.4 \times FCSV$$

3.3.3 Determine the **Inoculum Volume** by dividing the **Assay Sample Volume** by 20.

*For ease of inoculation, a sufficient quantity of 0.15 M Na_2HPO_4, pH 7.0 - 7.5, may be added to the **Inoculum Volume** to give a more usable working **Inoculation Volume** (e.g., 1.0 mL). For example, if an **Inoculum Volume** of 0.73 mL is to be placed onto 10 vessels, then 10.5 × (1 - 0.73 mL) = 2.84 mL of sodium phosphate, pH 7.0-7.5 could be added to 10.5 × 0.73 = 7.67 mL of concentrated sample. Each milliliter of the resulting mixture will contain the required **Inoculum Volume**.*

The **Inoculum/Inoculation Volume** should be no greater than 0.04 mL/cm^2 of the cell culture test vessel surface area. If the **Inoculum** or **Inoculation Volume** is greater than 0.04 mL/cm^2,use larger culture vessels.

3.4 Inoculate each BGM cell culture test vessel with an amount of assay control or water sample equal to the **Inoculum** or **Inoculation Volume** and record the date of inoculation on the Virus Data Sheet (see Appendix 2).

Avoid touching either the cannula or the pipetting device to the inside rim of the cell culture test vessels to avert the possibility of transporting contaminants to the remaining culture vessels.

3.4.1 Total Culturable Virus Assay Controls

Run a negative and positive assay control with every group of samples inoculated onto cell cultures.

(a) Negative Assay Control: Inoculate a BGM culture with a volume of sodium phosphate, pH 7.0 - 7.5, equal to the **Inoculum** or **Inoculation Volume**. This culture will serve as negative control for the tissue culture quantal assay. If any Negative Assay Control develops cytopathic effects (CPE), all subsequent assays of water samples should be halted until the cause of the positive result is determined.

(b) Positive Assay Control: Dilute a stock of attenuated poliovirus type 3 in sodium phosphate, pH 7.0 - 7.5, to give a concentration of 20 PFU per **Inoculum** or **Inoculation Volume**. Inoculate a BGM culture with an amount of diluted virus equal to the **Inoculum** or **Inoculation Volume**. This control will provide a measure for continued sensitivity of the cell cultures to virus infection. Additional positive control samples may be prepared by adding virus to a small portion of the final concentrated sample and/or by using additional virus types. If any Positive Assay Control fails to develop CPE, all subsequent assays of water samples should be halted until the cause of the negative result is determined.

3.4.2 Inoculation of Water or Solids Samples

*Samples from environmental waters and solids may contain components that are cytotoxic to cell cultures or may have virus levels too high to quantitate using undiluted samples. To avoid losing the entire sample to these conditions, this procedure calls for dividing the **Assay Sample Volume** into two equal subsamples and assaying one of the subsamples one week after the first.*

(a) Rapidly thaw one subsample, if frozen, and inoculate an amount equal to the **Inoculum** or **Inoculation Volume** onto each of ten cell cultures (see **Figure 15-2**).

Hold a thawed subsample for no more than 4 h at 4°C. Warm the subsample to room temperature just before inoculation. Freeze any remaining portion of the first subsample at -70°C for use with Step 3.4.2e.

Figure 15-2 Inoculation of 25 cm^2 Flasks.

(b) Determine if cell death has occurred due to cytotoxicity (see **Figure 15-3**). If the sample is cytotoxic, go to Step 3.4.2e. If there is no evidence for cytotoxicity, determine the number of flasks that are positive for CPE.

Examine cells, especially during the first three days, for the development of cytotoxicity. Determine cytotoxicity from the initial daily macroscopic examination of the appearance of the cell culture monolayer by comparing the negative control from Step 3.4.1a and the positive control from Step 3.4.1b with the test samples from Step 3.4.2. Cytotoxicity should be suspected when the cells in the test sample develop CPE within 24 h or before its development on the positive control.

*A small portion of the **Final Concentrated Sample** may by inoculated onto cultures several days before inoculating the first subsample as a control for cytotoxicity.*

(c) If at least three cell cultures are negative for CPE (see **Figure 15-3**),

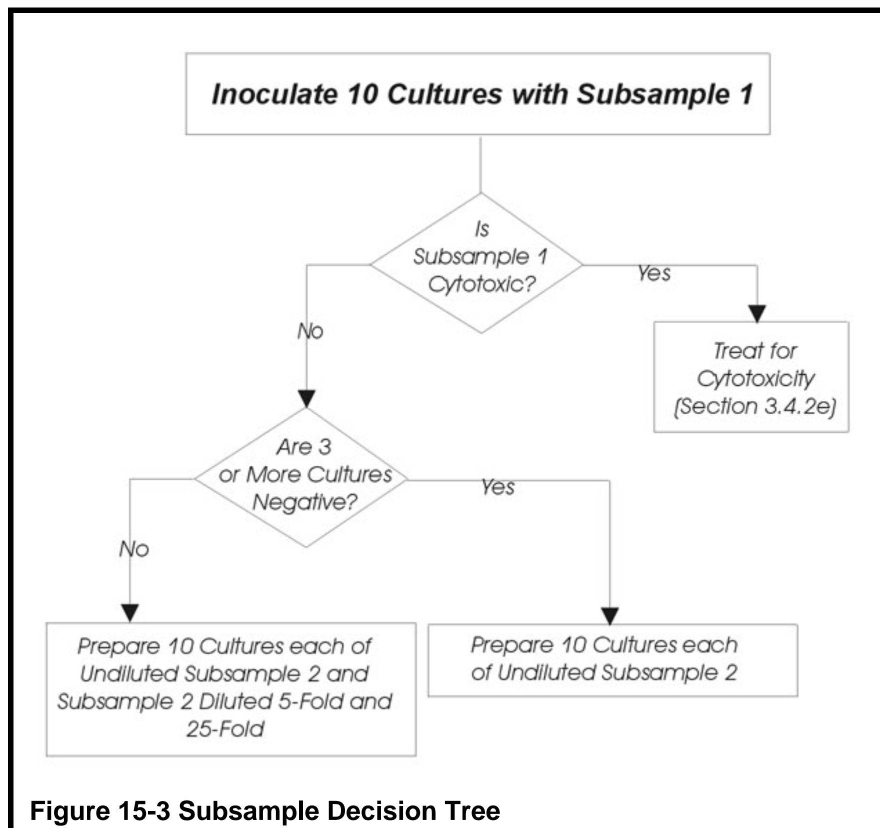


Figure 15-3 Subsample Decision Tree

thaw a second subsample and inoculate an amount equal to the **Inoculum** or **Inoculation Volume** onto each of ten additional BGM cell cultures.

(d) If more than seven cultures are positive for CPE after seven days (see **Figure 15-3**), prepare and inoculate BGM cell cultures with dilutions of the second subsample.

(d.1) Prepare a 1:5 dilution by adding a volume equal to 0.1334 × **Assay Sample Volume** (amount "a") to a volume of 0.15 M sodium phosphate (pH 7.0-7.5) equal to 0.5334 × **Assay Sample Volume** (amount "b"). If the more convenient **Inoculation Volume** is used, determine amount "b" by multiplying 0.5334 × **Assay Sample Volume** × (**Inoculation Volume** ÷ **Inoculum Volume**).

(d.2) Mix the 1:5 dilution thoroughly and then prepare a 1:25 dilution by adding amount "a" of the 1:5 diluted sample to amount "b" of 0.15 M sodium phosphate (pH 7.0-7.5).

(d.3) Using an amount equal to the **Inoculum** or **Inoculation Volume**, inoculate ten cell cultures each with undiluted subsample 2, subsample 2 diluted 1:5 and subsample 2 diluted 1:25, respectively.

Freeze the remaining portions of the 1:25 dilution at -70°C until the sample results are known. If the inoculated cultures are all positive, thaw the remaining 1:25 dilution and prepare 1:125, 1:625 and 1:3125 dilutions by transferring amount "a" of each lower dilution to amount "b" of sodium phosphate as described above. Inoculate 10 cultures each with the additional dilutions and freeze the remaining portion of the 1:3125 dilution. Continue the process of assaying higher dilutions until at least one test vessel at the highest dilution tested is negative. Higher dilutions can also be assayed along with the initial undiluted to 1:25 dilutions if it is suspected that the water to be tested contains more than 500 most probable number (MPN) of infectious total culturable virus units per 100 L.

A maximum of 60 and 580 MPN units per 100 L can be demonstrated by inoculating a total of 20 cultures with the undiluted ***Assay Sample Volume*** *or a total of 10 cultures each with undiluted sample and sample diluted 1:5 and 1:25, respectively.*

(e) If the first subsample is cytotoxic, prepare and inoculate BGM cell cultures with dilutions of the first subsample remaining from Step 3.4.2a.

(e.1) Prepare 1:2, 1:4 and 1:8 dilutions of the first subsample by serially diluting an amount equal to 1.3 to 5.3 times the **Inoculum** or **Inoculation Volume** with an equal volume of 0.15 M sodium phosphate (pH 7.0-7.5). Inoculate up to five cell cultures with each dilution using an amount equal to the **Inoculum** or **Inoculation Volume**.

With most water samples a 1:2 dilution should be sufficient to overcome cytotoxicity.

(e.2) If cytotoxicity is overcome by dilution, dilute the second subsample by the amount necessary to remove cytotoxicity and inoculate a number of cultures equal to ten times the dilution factor used using the **Inoculum** or **Inoculation Volume** (e.g., if a 1:2 dilution does not show cytotoxicity, inoculate 10 × 2 = 20 cell cultures).

If dilution fails to remove cytotoxicity, the procedures described in Chapter 8 (April 1986 revision) may be used.

Remaining portions of the second subsample should be frozen at -70°C to prepare additional dilutions if required due to high virus titers.

In addition to dilution, the changing of liquid maintenance medium at the first signs of cytotoxicity may prevent further development.

3.5 Rock the inoculated cell culture test vessels gently to achieve uniform distribution of inoculum over the surface of the cell monolayers (**Figure 15-4**). Place the cell culture test vessels on

a level stationary surface at room temperature so that the inoculum remains distributed evenly over the cell monolayer.

Figure 15-4 Distributing Inoculum

3.6 Continue incubating the inoculated cell cultures for 80 - 120 min to permit viruses to adsorb onto and infect cells.

It may be necessary to rock the vessels every 15-20 min or to keep them on a mechanical rocking platform during the adsorption period to prevent cell death in the middle of the vessels from dehydration.

3.7 Add liquid maintenance medium and incubate at 36.5 ± 1°C.

Warm the maintenance medium to 36.5 ± 1°C before placing it onto cell monolayers. Add the medium to the side of the cell culture vessel opposite the cell monolayer. Avoid touching any pipetting devices used to the inside rim of the culture vessels to avert the possibility of transporting contaminants to the remaining vessels. The cultures may be re-fed with fresh maintenance medium after 4 - 7 days.

3.8 Examine each culture microscopically for the appearance of CPE daily for the first three days (**Figure 15-5**) and then every couple of days for a total of 14 days.

CPE may be identified as cell disintegration or as changes in cell morphology (compare ***Figure 15-7*** *with* ***Figure 15-6****; Malherbe and Strickland-Cholmley, 1980). Rounding-up of infected cells is a typical effect seen with enterovirus infections. However, uninfected cells round-up during mitosis and a sample should not be considered positive unless there are significant clusters of rounded-up cells over and beyond what is observed in the uninfected controls.*

Figure 15-5 CPE Examination

3.9 Freeze cultures at -70°C when more than 75% of the monolayer shows signs of CPE. Freeze all remaining negative cultures, including controls, after 14 days.

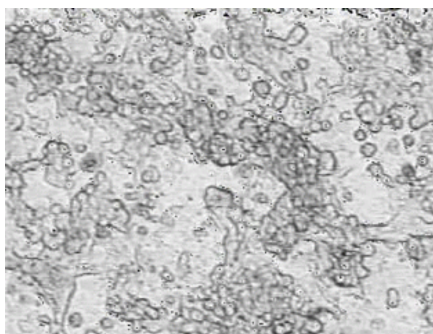


Figure 15-6 Culture Without CPE

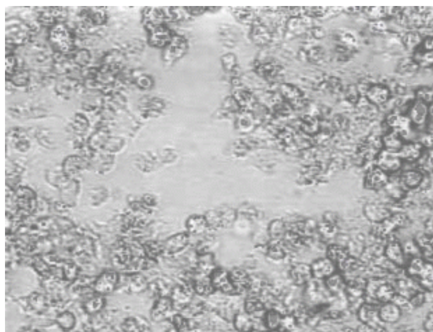


Figure 15-7 Culture With CPE

3.10 Thaw all the cultures to confirm the results of the previous passage. Filter at least 10% of the medium from each vessel that was positive for CPE or that appeared to be bacterially contaminated through separate 0.22 µm sterilizing filters (**Figure 15-8**). Then inoculate another BGM culture with 10% of the medium from the previous passage for each vessel, including those that were negative. Repeat Steps 3.7 - 3.8.

Confirmation passages may be performed in small vessels or multiwell trays, however, it may be necessary to distribute the inoculum into several vessels or wells to insure that the ***Inoculum*** *or* ***Inoculation Volume*** *is less than or equal to 0.04 mL/cm^2 of surface area.*

Figure 15-8 Filtration of Positive Samples

3.11 Score cell cultures that developed CPE in both the first and second passages as confirmed positives.

Cultures that show CPE in only the second passage must be passaged a third time along with the negative controls according to Steps 3.9 - 3.10. Score cultures that develop CPE in both the second and third passages as confirmed positives.

Cultures with confirmed CPE may be stored in a -70°C freezer for research purposes or for optional identification tests.[2]

4. Virus Quantitation

4.1 Record the total number of confirmed positive and negative cell cultures for each subsample onto a Total Culturable Virus Data Sheet (Appendix 3). Do not include the results of tests for cytotoxicity!

4.2 Transcribe the number of inoculated cultures and the confirmed number of positive cultures from the Total

[2]For more information see Chapter 12

Culturable Virus Data Sheet for each subsample to the Quantitation of Total Culturable Virus Data Sheet (Appendix 4). If dilutions are not required, add the values to obtain a total undiluted count for each sample.

4.3 Calculate the MPN/mL value (M_m), the upper 95% confidence limit/mL (CL_{um}) and the lower 95% confidence limit/mL (CL_{lm}) using the total undiluted count from step 4.2 and an MPN software program.[3] If dilutions are required, calculate the MPN/mL value and 95% confidence limits using only the values from the second subsample. Place the values obtained onto the Quantitation of Total Culturable Virus Data Sheet.

4.4 Calculate the MPN per 100 liter[4] value (M_l) of the original sample according the formula:

$$M_l = \frac{100\ M_m S}{D}$$

where S equals the **Assay Sample Volume** and D equals the **Volume of Original Water Sample Assayed** (the values for S and D can be found on the Virus Data Sheet). Record the value of M_l onto the Virus Data Sheet.[5]

4.5 Calculate the 95% confidence limits.

4.5.1 Calculate the lower 95% confidence limit per 100 liter value (CL_l) for each water sample according to the formula:

$$CL_l = \frac{100\ CL_{lm} S}{D}$$

where CL_{lm} is the lower 95% confidence limit per milliliter from the Quantitation of Total Culturable Virus Data Sheet.

4.5.2 Calculate the upper 95% Confidence Limit per 100 liter value (CL_u) according to the formula:

$$CL_u = \frac{100\ CL_{um} S}{D}$$

where CL_{um} is the upper 95% confidence limit per milliliter from the Quantitation of Total Culturable Virus Data Sheet.

4.5.3 Record the lower and upper limits per 100 liter values on the Virus Data Sheet (see Appendix 2 and **Figure 5.9**).

Figure 15-9 Recording Values

4.6 Calculate the total MPN value and the total 95% confidence limit values for each QC and PE sample by multiplying the values per milliliter by S and dividing by 0.4.

5. References

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Fout, G.S. 1999. Appendix H. Method for the recovery and assay of total cultural viruses from sludge. *In* Environmental Regulations and Technology: Control of Pathogens and Vector Attraction in Sewage Sludge. U.S. Environmental Protection Agency Publication No. EPA/625/R-92/013 (October, 1999

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[3]A MPN software program (MPNV) can be downloaded from http://www.epa.gov/microbes/

[4]Virus titers may be expressed in terms of MPN per volume other than 100 L by substituting the "100" value in the equations in Sections 4.4 - 4.5 with the volume desired. Virus titers from solids samples should be expressed on a per gram basis (e.g., MPN/4 g).

[5]Use significant figures when reporting all results throughout the protocol (see APHA, 1995, p. 1-17).

revision). Office of Research and Development, Washington, D.C.

Morris, R., and W.M. Waite. 1980. Evaluation of procedures for recovery of viruses from water-II detection systems. Water Res. **14**: 795-798.

Appendix 1. VENDORS

The vendors listed below represent one possible source for required products. Equivalent items from other vendors may substituted for items given in the text.

Costar Corp.
7035 Commerce Circle
Pleasanton, CA 94588
(800) 882-7711

Difco Laboratories
P.O. Box 331058
Detroit, MI 48232
(800) 521-0851 (Ask for a local distributor)

Life Technologies
8400 Helgerman Court
Gaithersburg, MD 20898
(800) 828-6686

PGC Scientifics
7311 Governors Way
Frederick, MD 21704
(800) 424-3300

Appendix 2. Virus Data Sheet

VIRUS DATA SHEET			
SAMPLE NUMBER:			
ANALYTICAL LABORATORY NAME:			
ANALYTICAL LABORATORY ADDRESS: CITY:		STATE:	ZIP:
ANALYST NAME:			
VOLUME OF ORIGINAL WATER SAMPLE ASSAYED (D):			L
ASSAY SAMPLE VOLUME (S):			mL
INOCULUM VOLUME:			mL
INOCULATION VOLUME (IF USED):			mL
DATES ASSAYED BY CPE:			
	1st Passage	2nd Passage	3rd Passage (If necessary)
First Subsample:			
Second Subsample:			
MPN/100 L[1]:		95% CONFIDENCE LIMITS/100 L LOWER:	UPPER:
COMMENTS:			

[1]Calculate MPN/100 L and Lower and Upper 95% Confidence Limits/100 L from the Quantitation of Total Culturable Virus Data Sheet as described in Sections 4.4 - 4.5.

Appendix 3. Total Culturable Virus Data Sheet

TOTAL CULTURABLE VIRUS DATA SHEET						
SAMPLE #:						
	Total Number of Replicates					
	First Subsample			Second Subsample		
Sample	Inoculated	Without CPE	With CPE	Inoculated	Without CPE	With CPE
1st Passage Neg. Cont.						
Pos. Cont.						
Undiluted						
1:5 Dil.						
1:25 Dil.						
2nd Passage[1] Neg. Cont.						
Pos. Cont.						
Undiluted						
1:5 Dil.						
1:25 Dil.						
3rd Passage[2] Neg. Cont.						
Pos. Cont.						
Undiluted						
1:5 Dil.						
1:25 Dil.						

[1]A portion of medium from each 1st passage vessel, including controls, must be repassaged for conformation. The terms "Undiluted," "1:5 Dilution" and "1:25 Dilution" under the 2nd and 3rd Passage headings refer to the original sample dilutions for the 1st passage. If higher dilutions are used, record the data from the three highest dilutions showing positive results and place the actual dilution amount in the sample column.

[2]Samples that were negative on the first passage and positive on the 2nd passage must be passaged a third time for conformation. If a third passage is required, all controls must be passaged again.

Comments:

Appendix 4. Quantitation of Total Culturable Virus Data Sheet

QUANTITATION OF TOTAL CULTURABLE VIRUS DATA SHEET

SAMPLE NUMBER:

Sample	Number Replicates inoculated	Number with CPE	MPN/mL[1]	95% Confidence Limits	
				Lower	Upper
Undiluted Samples					
Subsample 1					
Subsample 2					
Total Undiluted					
Subsample 2 results (Dilutions Required)					
Undiluted					
1:5 Dilution					
1:25 Dilution					

[1]Use the values recorded in the Total Undiluted row to calculate the MPN/mL result and confidence limits when dilutions are not required. If dilutions are required, base the calculation upon the values recorded in the Undiluted, 1:5 Diluted and 1:25 Diluted rows for subsample 2. If higher dilutions are used for subsample 2, record the data from the three highest dilutions showing positive results and place the actual dilution amount in the sample column.